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TITLE: P53 Mutation Analysis to Predict Tumor Response in  
Patients Undergoing Neoadjuvant Treatment for Locally  
Advanced Breast Cancer

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## Introduction

The two most effective classes of chemotherapeutic drugs in breast cancer are the anthracyclines and the taxanes, which differ in mechanisms of action and resistance. Responsiveness to anthracyclines and taxanes may be mediated in part by the p53 mutational status of the tumor. P53 mutation status has had limited usefulness as a predictive tumor marker given the technical complexity of previous methods to determine it, however the development of p53 GeneChip technology has made high-throughput mutation analysis more feasible. This technology has been successfully applied to human tumor specimens<sup>1,2</sup>. Dr. Conway Dorsey's laboratory has previously determined the spectrum of expected p53 mutations in breast cancer<sup>3</sup> using sequencing, and is performing the GeneChip analysis and sequencing in this study.

An ongoing multiinstitutional prospective trial, breast cancer patients who are receiving neoadjuvant chemotherapy have serial response assessments performed and undergo sampling of their tumor for research purposes at three time points. These timepoints are: 1) prior to any chemotherapy, 2) following treatment with an anthracycline-containing regimen. Those that receive a subsequent chemotherapy have another sample obtained after that regimen. This project involves analyzing the banked specimens for p53 mutation status using the GeneChip method. Specific mutations identified will be further examined for functional impact in a yeast-based assay to be compared with the clinical response. We hypothesize that p53 status of the primary tumor will predict response to anthracycline-based and taxane-based chemotherapy given at different times in the same patient.

## **Body**

This award is for performance of laboratory assays upon banked tumor specimens obtained from ongoing correlative science trials that are funded through alternative mechanisms. The performance of those trials, however, is crucial to the outcome of this project, so is summarized here. The trials include Lineberger Comprehensive Cancer Center (LCCC) Project 9819 and a multiinstitutional trial, Cancer and Leukemia Group B (CALGB) Protocol 150007, which is a joint effort of CALGB, the American College of Radiology Imaging Network (ACRIN), and the National Cancer Institute Specialized Programs of Research Excellence (SPORE). These trials are both open and enrolling patients. All participating patients are required to have received an anthracycline and/or taxane-based chemotherapy and have ascertainment of clinical and pathologic response to therapy in order to be included. LCCC 9819 at this time has enrolled 71 patients, and the CALGB/ACRIN/InterSPORE trial 139 patients.

## **Statement of Work**

Progress upon the approved statement of work is outlined below in the format used in the original application.

### *Task 1. To optimize the GeneChip method of p53 mutation analysis in the UNC Molecular Epidemiology Core Laboratory (months 1-6)*

#### **Optimization of GeneChip method**

This portion of the research involved the establishment of multiplex PCR conditions to co-amplify all p53 exons from within one reaction, and the optimization of the p53 GeneChip hybridization conditions and analysis of microarray data. The p53 GeneChip assay has been optimized using the Affymetrix normal control DNA (human placental DNA) and cell lines (BT549, Bt474, MDA-MB-231, and MDA-MB-435), and has been successfully applied to human breast cancer core biopsy specimens.

Because of the very small quantities of DNA expected from the breast core biopsies, our first priority in establishing assay conditions for the p53 GeneChip assay was to determine the smallest amount of DNA that could be reasonably amplified from the cores, but that would provide a valid p53 mutation result. This is crucial because only 8-10mg of total tissue is obtained from each core biopsy, with nucleic acid-based studies planned by several collaborating laboratories. Using the above cell lines, the DNA concentration required for the multiplex p53 PCR reaction, which amplifies each p53 exon 2-11 in individual fragments, has been successfully reduced from an original amount of 250ng DNA down to 50ng DNA. This reduction of DNA has not compromised our ability to identify mutations within cell line DNAs. The research plan includes single strand conformational polymorphism (SSCP) and sequencing analysis to comprehensively identify p53 mutations in GeneChip-negative samples. This technique of large-fragment PCR upon these limited tissue samples will allow the SSCP and mutation analysis to be performed with a minimal of required DNA. The optimization of the GeneChip method was completed in 2003.

Task 2. To determine the p53 mutational status of the primary breast cancers before any chemotherapy. In cases whose tumors exhibited p53 mutations pre-chemotherapy, determine if the same mutations are detectable after anthracycline then again after taxane with or without trastuzumab (Months 6-36).

### **Nucleic acids processing from core biopsies.**

Dr. Conway-Dorsey and Dr. Perou are performing complementary assays (p53 mutation analysis and gene expression array, respectively) upon the frozen tissue in the UNC institutional trial. In the multiinstitutional trial, Dr. Joe Gray (UCSF) and Chris Haqq (UCSF) are also performing comparative genomic hybridization and additional RNA-based assays, so it has been crucial to optimize the nucleic acid retrieval method for this study. For this reason, a great deal of effort has been made to minimize the tissue, DNA, and RNA needs of each group so that all the planned assays may be performed. In order to optimize the conditions for maximal nucleic acid retrieval from these limited tissue resources, a training set of biopsies was obtained and tested. Several approaches to maximize RNA and DNA acquisition from core biopsies have been tested, and we have found that the optimal method remained the simplest. In this schema, tumor enrichment is performed by examination of an H&E-stained longitudinal section followed by manual dissection of non tumor-containing areas of the core. The remaining portion is divided evenly and processed for RNA and DNA. The first cohort of frozen cores from the UNC institutional trial (LCCC 9819) have been processed in this manner, and both test cores and study cores from the multiinstitutional trial have been similarly processed. All UNC samples were estimated to have >50% tumor after enrichment.

The DNA from these samples has been provided to the Molecular Epidemiology lab. These are summarized in Table 1. In November 2003, we received 14 DNA samples derived from 5 test cores from UCSF. In June 2004, 20 additional test core samples were received from UCSF. Two groups of DNA samples have recently been received from study cores. In October 2004, 15 DNA samples were received from UNC trial 9819. The DNAs for this set of cores were prepared by Dr. Chuck Perou's laboratory at UNC. Also in October 2004, 6 DNA samples were received from UCSF for the multiinstitutional CALGB/ACRIN/InterSPOR trial. The DNA samples were all of good quality and produced abundant product in the p53 exon 2-11 multiplex PCR which is the first step in the p53 GeneChip analysis, with the exception of UNC 9819 sample 02-0106-B4 which yielded minimal product.

All of the test cores and study cores processed at UCSF thus far have yielded at least 1.5 ug DNA, sufficient for both the p53 mutation analyses (500 ng requested) and the comparative genomic hybridization studies being performed in Dr. Joe Gray's lab (1ug requested). The cores processed from the UNC 9819 Study in Dr. Perou's lab yielded on average about 1ug DNA. The DNA quantity used for each multiplex p53 PCR reaction was 50ng.

The p53 GeneChip microarray assay is in progress for all of these core DNAs. The GeneChip assay is designed to detect point mutations and single base deletions in exons 2-11 of p53. We expect to have results for these samples the last week on October. Additionally, because this phase of p53 screening is very rapid, we anticipate being able to carry out p53 GeneChip analysis on all banked study core DNA samples within the next two months.

### **p53 Analysis to Detect Larger Deletions and Insertions**

Core DNA samples that are negative for a p53 mutation by the GeneChip assay will be subsequently screened for insertions and deletions greater than 1 base pair using single strand conformational polymorphism (SSCP) and sequencing. We have previously developed and extensively used SSCP and sequencing to detect mutations in p53 exons 4-8. For the present study, we have optimized a large, single fragment PCR reaction to amplify the entire coding region of p53 from the large molecular size DNA obtained from the frozen cores, and this will serve as template for SSCP and sequencing. We have also optimized the SSCP/sequencing methods for the additional exons of p53: exons 2, 3, 9, 10, and 11, and have identified positive control cell lines for SSCP that contain known p53 mutations in each of these exons.

Task 3. To correlate p53 status with response to anthracycline chemotherapy, then taxane with or without trastuzumab in the same patient (months 30-36):

This aim begins in year 3.

Task 4. To compare p53 status with results of other planned assays within the larger correlative science trial such as bcl-2, estrogen receptor, and gene expression array analysis (months 1-36).

In the upcoming year, we examine the banked specimens for p53 point mutations and single base deletions using the Affymetrix GeneChip. This will allow performance of the planned correlations with other biomarkers. Notably, in its role as the supporting institution for the prospective trials, the National Cancer Institute has initiated the development of a database that will allow multiple investigators involved in this study to cross-examine the relationships of various markers to each other and to outcome.

Task 5. To functionally classify the p53 mutants identified in breast cancer using established and newly developed yeast-based assays (Months 12-36).

### **Development of a versatile yeast-based system for construction and functional characterization of tumor-associated p53 mutations.**

A recently developed system for *in vivo* site-directed mutagenesis by oligonucleotides was adapted to allow rapid construction in yeast of any p53 mutant of choice<sup>4</sup>. To this aim we developed a panel of seven isogenic "p53-host" yeast strains containing the human wild type p53 cDNA under control of the inducible *GAL1* promoter integrated at the *TRP5* locus on chromosome VII. The p53 cDNA has been interrupted at different position in each of the seven strains by inserting one ICORE cassette. The cassette comprises, in addition to a COUNTER-selectable *KLURA3* and the RREPORTER *KanMX4*, the I-SceI endonuclease gene under control of the *GAL1* promoter and one copy of its unique 18nt recognition site. Thus, induction of I-SceI when cells are grown on galactose leads to the generation of a single DNA double strand break at the ICORE cassette, resulting in a dramatical increase in homologous DNA targeting along with coincident loss of the cassette. The ICOREs (one per p53-host strain) were positioned at 90 nucleotide intervals in the p53 cDNA with the

proximal being introduced at nucleotide 400 and the most distal at nucleotide 940. Each ICORE provides a 48-nt window for mutagenesis on each side of the integration. Thus, the panel of seven strains can be used to generate any p53 mutations between amino acid 118 and 329 (Jordan *et al.*, unpublished).

To create p53 mutants, ICORE cassettes can be replaced using oligonucleotides that contain the desired mutation and have homology for both ICORE integration sites (see Table 1), according to the *delitto-perfetto* protocol<sup>4</sup>. A "p53-host" strain can then be rapidly mated with a large panel of "p53-reporter" strains to characterize the functional impact of the chosen p53 mutations<sup>5,6</sup>. Diploid cells are selected, purified and used in transactivation assays. Two sets of p53-reporter strains are available: one is based on the *ADE2* gene, that provides for a simple qualitative functional assay based on colony color; the second is based on the quantitative firefly luciferase gene. Many different p53 response elements (REs) have been placed upstream of the reporter genes resulting in a wide range of p53-dependent transactivation. The ability to tightly regulate p53 protein expression using the rheostatable *GAL1* promoter combined with the use of REs with different strength provides a robust tool to characterize p53 alleles with altered transactivation functions.

#### **Functional characterization of p53 mutations associated with breast cancer.**

Thirty-one missense p53 mutations associated with breast cancer were chosen for functional analysis (see Figure 1). Different criteria were applied in the selection including structure/function relationship (*e.g.*, location in zinc finger region and loops L2 or L3), frequency in breast and other cancers (derived from the IARC p53 mutation database), occurrence in the germline (*e.g.*, Li-Fraumeni and Li-Fraumeni-like syndromes), association with cancer predisposition diseases (BRCA-1-associated breast cancer), and unavailability of functionality data.

Twenty-two mutations have been studied so far in the *ADE2*-based visual reporter system using ten different REs. Seven mutations ( $7/22 = 32\%$ ) retained partial functionality towards at least a subset of REs (see Figures 2 and 3). Three of these have been reported in Li-Fraumeni families. One partial function mutant (L194P) also showed a change in transactivation specificity. The analysis with the visual assay will be completed for the remaining 9 mutants and we will then use the quantitative luciferase-based assay to characterize in more detail the partial function mutants.

Once the molecular analysis of the p53 status from the core biopsies becomes available, the experimental protocol we developed will allow us to rapidly construct and test p53 mutations for which functional data is not yet available. The final goal of our study will be to evaluate whether functionality data add prognostic value to the analysis of p53 status in breast cancer patients undergoing the neoadjuvant therapy protocol.



Table 1. Summary of Breast Core DNA Samples Received for p53 Mutation Analysis

CALGB/ACRIN/InterSPORE <u>Test</u> Core Biopsy DNA Samples				
Received - June 2004				
#	Sample #	DNA Received (ng)	Multiplex PCR Amplification	GeneChip Assay
1	UNC 003-0072 OCT 1	500	excellent	In progress
2	UNC 003-0077 OCT 2	500	excellent	In progress
3	UNC 2/17/04 OCT-3	500	excellent	In progress
4	B1200	500	excellent	In progress
5	B1204	500	excellent	In progress
6	B1222	500	excellent	In progress
7	B1220	500	excellent	In progress
8	UNC 2/17/04 OCT-5	500	excellent	In progress
9	UNC 2/17/04 OCT-6	500	excellent	In progress
10	B1226	500	excellent	In progress
11	B1238	500	excellent	In progress
12	B1240	500	excellent	In progress
13	B1247-1	500	excellent	In progress
14	B1247-2	500	excellent	In progress
15	B1247-4	500	excellent	In progress
16	B1247-6	500	excellent	In progress
17	B1251-1 (A)	500	excellent	In progress
18	B1251-2 (B)	500	excellent	In progress
19	B1251-3 (C)	500	excellent	In progress
20	B1251-4 (D)	500	excellent	In progress

CALGB/ACRIN/InterSPORE Study Core Biopsy DNA Samples

Received - October 2004

#	Sample #	DNA Received (ng)	Multiplex PCR Amplification	GeneChip Assay
1	89800 F2 (UCSF)	500	excellent	In progress
2	89800 F4 (UCSF)	500	excellent	In progress
3	89867 F1 (UCSF)	500	excellent	In progress
4	89999 F2 (UPENN)	500	excellent	In progress
5	89999 F3 (UPENN)	500	excellent	In progress
6	90128 F1 (UPENN)	500	excellent	In progress

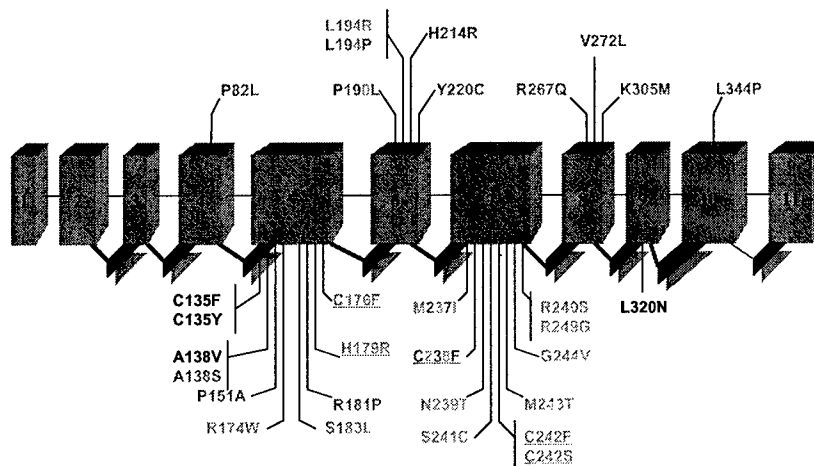
UNC LCCC9819 Study Core Biopsy DNA Samples

Received - October 2004

#	TPF Sample #	Total DNA Yield (ng)	Multiplex PCR Amplification	GeneChip Assay
1	02-0053-B3	1000.8	excellent	In progress
2	02-0106-B4	500	(marginal)	In progress
3	02-0115-B2	1002	excellent	In progress
4	01-0134-B	1002.5	excellent	In progress

5	00-0129B	1020	excellent	In progress
6	00-0315B	1008	excellent	In progress
7	00-0392B	1018.8	excellent	In progress
8	00-0426B	1045	excellent	In progress
9	01-0080B	741	excellent	In progress
10	01-0249B	1000.8	excellent	In progress
11	01-0262B	995.5	excellent	In progress
12	01-0319B	1009.8	excellent	In progress
13	01-0372B	1038.8	excellent	In progress
14	01-0432B(1)	1210	excellent	In progress
15	01-0514B	1023	excellent	In progress

Figure 1. Examination of breast cancer-related p53 mutations

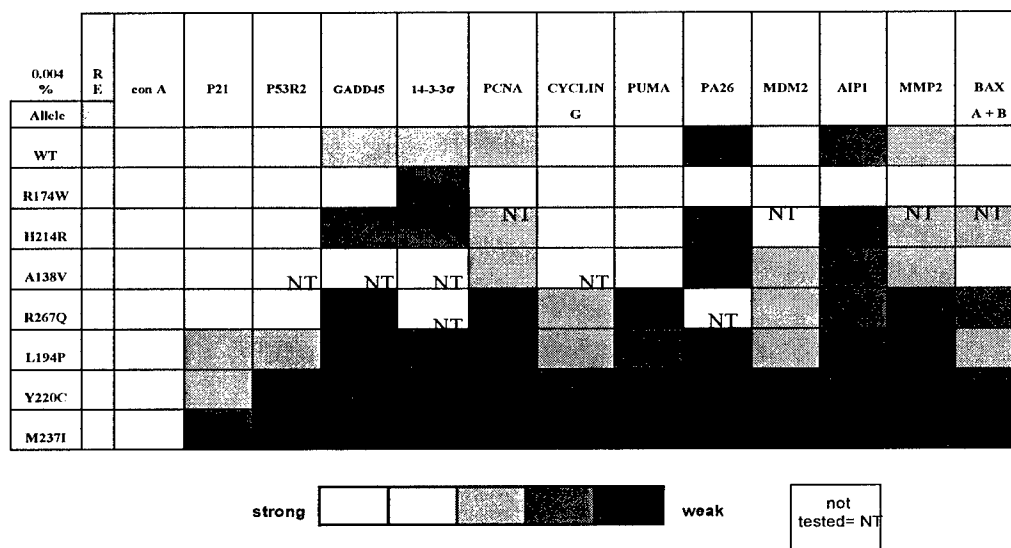


- also found among LFS, LFL, or familial
- in L2, L3, &/or Zinc Binding site
- associated with familial BRCA1 cancer
- different missense mutations at same codon

Figure 2. At high expression 7/22 (32%) p53 missense mutants associated with breast cancer are able to transactivate efficiently several REs in the yeast reporter system

	R E	con A	P21	P53R2	GADD45	14-3-3 $\sigma$	PCNA	CYCLIN G	PUMA	PA26	MDM2	AIP1	MMP2	BAX A + B
Allele														
WT		W	W	W	W	W	W	W	W	W	W	W	W	W
C135F														
C135Y														
A138V		W	W	W	W	W	W	W	W	A	W	W	W	W
A138S														
R174W		W	W	W	W	W	W	W	W	W	W	W	W	W
C176F														
L194P		W	W	W	A		A	W	W		W	A	W	W
L194R														
H214R		W	W	W	W	W	W	W	W	W	W	W	W	W
Y220C		W	W		W									
M237I		W	A	A										
C238F														
N239T														
S241C														
C242F														
C242S														
M243T														
G244V														
R249G														
R249S														
R267Q		W	W	W	W	W	A	W	W	W	W	W	W	W
V272L														

Figure 3. The mutants that are wild type for transactivation at high expression (2% galactose) are defective for transactivation from at least some REs at low expression (0.004% galactose).



## **Key Research Accomplishments**

### **Previously reported (2002-2003)**

- Establishment of conditions for Affymetrix GeneChip assay using frozen breast cancer tissue as starting material as well as cell line DNA. Test samples have identified p53 mutations in frozen tumors.
- Scaling down DNA requirements. The recommended amount of DNA for the GeneChip is 250 ng. In Dr. Dorsey's laboratory, conditions have been optimized for successful assay using only 50ng
- Demonstration of successful p53 amplification within 1-2 large fragments (1.5 kb) for PCR. This will allow performance of single strand conformational polymorphism (SSCP) and sequencing analysis in GeneChip-negative samples in spite of small amount of DNA available from these limited tissue resources.

### **Current year (2003-2004)**

- Optimization of RNA and DNA processing from core biopsies of breast tumors.
- Successful p53 exon 2-11 multiplex PCR in initial cohort of test and study core biopsies.
- Optimization of large, single fragment PCR reaction to amplify entire coding region of p53 from large molecular weight DNA
- Optimization of SSCP/sequencing methods for additional exons 2, 3, 9, 10, and 11.
- Identification of positive control cell lines for SSCP that contain known p53 mutations in each of exons 2, 3, 9, 10, and 11.
- Adaptation of a system for in vivo site-directed mutagenesis by oligonucleotides to allow rapid construction in yeast of any p53 mutant (Storici et al. PNAS 2003)
- Development of seven isogenic p53 host yeast strains that can be used to generate any p53 mutation between amino acid 118 and 329
- Development of method to mate p53 host strain with p53 reporter strain allowing characterization of functional impact of chosen p53 mutation (Inga A et al, MCB 2003; Resnick MA and Inga A. PNAS 2003)

## **Reportable Outcomes**

Previously reported (2002-2003)

none

Current (2003-2004)

- In vivo site-directed mutagenesis system adapted to allow rapid construction of defined p53 mutants in yeast (Storici et al. PNAS 2003)
- Method of characterization of p53 alleles with altered transactivation function (Inga A et al. MCB 2003; Resnick MA and Inga A. PNAS 2003)

## Conclusions

Ascertainment of tumor samples from patients undergoing neoadjuvant chemotherapy for locally advanced breast cancer is continuing. In addition to the UNC samples, the NCI-supported multiinstitutional study began in the fall 2002, and has already accrued \*\*\* patients. This confirms that the investigators will have the tissue resources to perform the planned analysis of p53 as a predictive marker in breast cancer. At the current rate of accrual, the prospective trials will complete on time. Preliminary data from test core biopsies from non-protocol patients suggests that the planned assays that will complement the p53 analysis are feasible despite the small amount of tissue available.

The GeneChip method of p53 mutation analysis in human tumors has been optimized in Dr. Conway Dorsey's laboratory. Moreover, her laboratory has successfully reduced the required amount of DNA to 50 ng, and has performed multiplex PCR amplification upon both test and study core biopsies with good results. The screening method for GeneChip-negative samples using SSCP and sequencing has been optimized for the entire coding region of p53 from the large molecular weight DNA obtained from core biopsies, and have identified positive cell line controls for mutations in each exon.

Once mutations are identified in the tumors from patients undergoing neoadjuvant chemotherapy, the specific mutations will be provided to Dr. Resnick in order to correlate clinical response to therapy with functional evaluation of the effect of these mutations upon transactivation in a yeast-based transactivation assay. Dr. Resnick's laboratory has adapted their in vivo site-directed mutagenesis method to allow rapid construction of p53 mutations of choice. They have also demonstrated the ability to characterize p53 alleles with altered transactivation functions.



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